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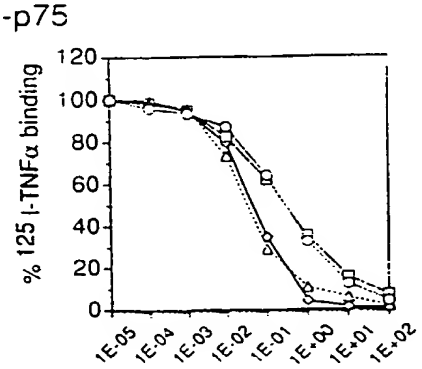
(54) Tumor necrosis factor muteins.

(57) Human TNF muteins having higher binding affinity for human p75-TNF receptor than for human p55-TNF receptor include muteins having at least one deffect amino acid relative to wild-type human TNF at a position corresponding to position 33, 65, 67, 75, 87, 143, 145 or 147 of the wild-type amino acid sequence.

Such muteins can be used to characterise human p75-receptor and, together with known muteins with selective binding affinity for human p55-TNF receptor, can aid in distinguishing between TNF functions mediated via binding to human p75-receptor and those mediated via binding to human p55-receptor.

FIG. 4



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The present invention relates to Tumor Necrosis Factor Muteins.

Tumor Necrosis Factor, or more specifically Tumor Necrosis Factor-alpha (for ease of reference, unless otherwise indicated, "Tumor Necrosis Factor" or "TNF" when used herein refers to TNF-α), is a cytokine primarily produced by stimulated macrophages. It exhibits not only a striking cytotoxicity against various tumour cells [Carswell et al., Procd. Nat. Acad. Sci., USA 72, 3666-3670, (1975)] but also plays a multiple role as a mediator of inflammation and the immune response [for an overview see Beutler and Cerami, Ann. Rev. Immunol. 7, 625-655 (1989); Bonavista and Granger (eds.) "Tumor Necrosis Factor: Structure. Mechanism of Action. Role in Disease and Therapy, Karger, Basel (1990)]. The primary structure of human Tumor Necrosis Factor-alpha (hTNF-α) has been deduced from the nucleotide sequence of a cDNA which has been cloned and expressed in E. coli [Pennica et al., Nature 312, 724-729 (1984); Marmenout et al., Europ. J. Biochem. 152, 515-522 (1985); Wang et al., Science 228, 149-154 (1985); Shirai et al., Nature 313, 803-806 (1985)]. A striking homology in amino acid sequence (30%) was found between hTNF-α and human Lymphotoxin, often referred to as human Tumor Necrosis Factor-beta (hTNF-β), a cytokine mainly produced by activated lymphocytes [Gray et al., Nature 312, 721-724 (1984); Fiers et al., Cold Spring Harbour Symp. 51, 587-595 (1986)].

hTNF-α with modified amino acid sequences, so called TNF-α-muteins, have also been described in various publications - for example Yamagishi et al., Protein Engineering 3, 713-719, (1990); Fiers in "Tumor Necrosis Factors: Structure, Function and Mechanism of Action"; Fiers et al. in Bonavista and Granger, pp. 77-81 (see above); Goh et al., (1991) entitled "Structural and functional domains in human tumor necrosis factors." Prot. Engineering 4: 385-389; Kircheis et al., (1992) entitled "Biological activity of mutants of human tumor necrosis factor-alpha," Immunology 76: 433-438; Van Ostade et al., (1991) entitled "Localization of the active site of human tumor necrosis factor (hTNF) by mutational analyses," EMBO J. 10: 827-836; Van Ostade et al., (1993) entitled "Human TNF mutants with selective activity on the p55 receptor," Nature 361: 266-269; Zhang et al., (1992) entitled "Site-directed mutational analysis of human tumor necrosis factor-α receptor binding site and structure-functional relationship," J. Biol. Chem. 267: 24069-24075; and in Ito et al., (1991) entitled "Novel muteins of human tumor necrosis factor alpha," Biochim. Biophys. Acta 1096: 245-252. In addition TNF-α-muteins have been the object of several patent applications, e.g. International Patent Applications Publ. Nos. WO 86/02381, WO 86/04606, WO 88/06625 and European Patent Applications Publ. Nos. 155,549; 158,286; 168,214; 251,037 and 340,333, and Deutsche Offenlegungsschrift Nr. 3843534.

Muteins of Lymphotoxin have also been disclosed in the art, e.g. in European Patent Applications Publ. Nos. 250,000; 314,094 and 336,383, as well as in the following two publications: Goh et al., (1991) entitled "Aspartic acid 50 and tyrosine 108 are essential for receptor binding and cytotoxic activity of tumor necrosis factor beta (lymphotoxin)," Prot. Engineering 4: 785-791 and Wakabayashi et al., (1990) entitled "Deletion of lysine 89 enhances the cytotoxicity and the receptor binding affinity of human lymphotoxin," J. Biol. Chem. 265: 7604-7609.

The biological effects of TNF are mediated via specific receptors, namely a receptor with an apparent molecular weight of 55 kD on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (p55-TNF-R) and a receptor with an apparent molecular weight of 75 kD on SDS-PAGE (p75-TNF-R). Both forms of TNF-receptors have been cloned, namely p55-TNF-R by Loetscher et al. [Cell 61, 351-359, (1990)] and p75-TNF-R for example by Dembic et al. [Cytokine 2, 53-58, (1990)] (for both receptors see also European Patent Application No. 90116707.2) and it was found more recently that both receptors bind not only TNF-α but also TNF-β with high affinity [Schönfeld et al., J. Biol. Chem. 266, 3863-3869 (1991)].

It is well known in the art that on the basis of its biological activities TNF-α can be a valuable compound for the treatment of various disorders. For example TNF-α, alone or in combination with interferon, can be an effective antitumor agent [Brouckaert et al., Int. J. Cancer 38, 763-769 (1986)]. However, its systemic toxicity is a major limitation to its wider therapeutic use [Taguchi T. and Sohmura Y., Biotherapy 3, 177-186 (1991)].

hTNF-α and mTNF-α bind with almost equal affinity to human p55-TNF-R and to human p75-TNF-R. It has, however, been shown that in mice human TNF-α (hTNF-α), only binds to the smaller mouse TNF receptor (murine p55-TNF-R). In mice hTNF-α is far less toxic than murine TNF-α (mTNF-α), which binds to both mouse receptors, mp55-TNF-R and mp75-TNF-R. For example, in C57B16 mice, the LD50 is about 10µg/mouse and 500µg/mouse with mTNF-α and hTNF-α, respectively [Brouckaert et al., Agents and Actions 26, 196-198 (1989); Everaerdt, B. et al., Biochem. Biophys. Res. Comm. 163, 378-385 (1989); Lewis, M. et al., Proc. Natl. Acad. Sci. USA 88, 2830 (1991); Brouckaert, P., Libert, C., Everaerdt, B. and Fiers, W. (1992). "Selective species specificity of tumor necrosis factor for toxicity in the mouse." Lymphokine Cytokine Res. 11, 193-196]. Hence it was proposed that the p75-TNF-R plays a special role in systemic toxicity.

It also has been reported that proliferative signals can be mediated by hp75-TNF-R in human T lymphocytes (Gehr et al., J. Immunol. 149, 911, 1992; Tartaglia et al., Proc. Natl. Acad. Sci. USA 88, 9292. 1991).

Human Tumor Necrosis Factor muteins, showing a significant difference between their binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor (hp75-TNF-R) and to the human p55-Tumor-Necrosis-Factor-Receptor (hp55-TNF-R), have been described in European Patent Application Publication No. 486 908, where hTNF muteins are disclosed which have retained the binding activity to hp55-TNF-R, but have lost nearly all binding to hp75-TNF-R.

According to the present invention there is provided a human Tumor Necrosis Factor mutein having higher binding affinity for human p75-Tumor-Necrosis-Factor-Receptor than for human p55 Tumor-Necrosis-Factor-Receptor (the term "human Tumor Necrosis Factor Mutein" when used herein includes pharmaceutically acceptable human Tumor Necrosis Factor Mutein salts).

The amino acid sequence of (wild-type) human TNF-α as disclosed by Pennica et al. [see above] is as follows:

```
 1                                                  10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

                    20                                                  30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

                                                   40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

                    50                                                  60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

                                                   70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

                    80                                                  90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

                                                  100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

                   110                                                 120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

                                                  130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU

                   140                                                 150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL


                                                                      157
                    TYR PHE GLY ILE ILE ALA LEU
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or as disclosed by Marmenout et al. (see above) or Wang et al. (see above) or Shirai et al. or more specifically as coded for by the nucleotide sequence of the insert of the plasmid pDS56/RBSII,SphI-TNFα - (SEQ ID No. 1; see Figures 1a and 1b and Example I; or Figures 3b1-3b3 of EP 486 908) coding for mature TNF-α.

Prior to the present invention there was no indication that hTNF muteins could be prepared which bind selectively to hp75TNF-R. Muteins according to the present invention can advantageously be used to characterise hp75-TNF-R and also have potential beneficial diagnostic and therapeutic applications, as will be described later.

Preferably the mutein comprises at least one amino acid change relative to wild-type human TNF-α at a position corresponding to position 33, 34, 65, 67, 75, 143, 145 and/or 147 of the wild-type sequence, when measured relative to the N-terminal amino acid. The term "corresponding to" is used herein to indicate that the muteins of the present invention need not be exactly homologous with wild-type human TNF-α at positions other than those indicated above, since at such positions deletions, insertions or substitutions are contemplated relative to the wild-type amino-acid sequence, provided that these have no substantial effect on binding affinity to hp75-TNF-R.

Amino acid substitutions in proteins and polypeptides which do not essentially alter biological activity are known in the art and described, e.g. by H. Neurath and R.L. Hill in "The Proteins", Academic Press, New York (1979), in particular in fig. 6 of page 14. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly and vice versa.

Preferably the mutein comprises at least one of the following amino acid changes at a position corresponding to the position indicated for the wild-type sequence:

A33T
K65A
K65W
Q67K
Q67T
Q67Y
L75H
L75W
D143N
D143E
D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145D
A145G
A145H
A145K
A145F
A145S
A145T
A145W
A145Y
A145V
E146R
S147L

In this nomenclature the letters used represent amino acids, based upon the single letter amino acid code. Dashes are used to separate amino acid changes at more than one position. For each amino acid change indicated, the first letter refers to the amino acid in wild-type human TNF-α while the second letter indicates the corresponding amino acid in the mutein. The numbers used indicate the positions in the wild-type sequence at which the amino acids indicated for the wild-type sequence occur.

Of these variants, those listed below are preferred, having been found to have particularly good binding selectivity for hp75-TNF-R:

K65W
D143N
D143E

D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145H
A145K
A145F
A145W
A145Y

Particularly preferred variants are those listed below:

D143N
D143E
D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145K
A145F
A145W
A145Y

It is notable that all of these latter alternatives have amino acid changes at positions corresponding to positions 143 and/or 145 of the wild-type sequence. Changes at these positions are therefore preferred.

The hTNF muteins of the present invention may additionally contain sequences of several amino acids which are coded for by "linker" sequences. These sequences may arise as a result of the expression vectors used for expression of the hTNF muteins as defined above.

The hTNF muteins of the present invention can also contain specific sequences that bind with high selectivity to an affinity carrier material so as to aid purification. Examples of such sequences are sequences containing at least two adjacent histidine residues (see in this respect European Patent Application, Publication No. 282 042). Such sequences bind selectively to nitrilotriacetic acid nickel chelate resins (Hochuli and Döbeli, Biol. Chem. Hoppe-Seyler 368, 748 (1987); European Patent Application, Publication No. 253 303). hTNF muteins which contain such a specific sequence can be linked either to the C-terminus or the N-terminus, or to both termini, of the hTNF-mutein amino acid sequences.

The hTNF muteins of the present invention can also be combined with different immunoglobulin heavy chain or light chain polypeptides. This leads to chimeric hTNF mutein immunoglobulin polypeptides which could have increased half-life in vivo. Increased half-life in vivo has been shown, e.g., for chimeric polypeptides consisting of the first two domains of the constant regions of the heavy chain or the light chain of a mammalian immunoglobulin (see Traunecker et al., Nature 331, 84-86 [1988] and European Patent Application, Publication No. 394 827). Chimeric proteins of hTNF muteins fused to any other peptide sequence are also possible.

The hTNF muteins can also be coupled to polymers, e.g. polyethylene glycol or polypropylene glycol having a molecular weight of 500 to 20,000 daltons (pegylated hTNF-muteins). This leads to protected hTNF mutein compositions which could be substantially non-immunogenic. Several modes of coupling the polymer with the polypeptide are available and described, e.g., in U.S. Patent No. 4.179.337. Accordingly a pegylated hTNF-mutein or a pharmaceutically acceptable salt thereof is also an object of the present invention.

The hTNF muteins of the present invention can be produced by methods known in the art and described e.g. in Sambrook et al. [Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbour Laboratory Press, USA (1989)] or e.g. in the following paragraphs. Whether such hTNF muteins still show selective binding affinity for the p75-TNF-R can be determined as described in the following Examples. Alternatively, the hTNF muteins of the present invention can also be chemically

synthesized using standard methods known in the art, preferably solid state methods, such as the methods of Merrifield (J. Am. Chem. Soc. 85, 2149-2154 [1963]). Furthermore pharmaceutically acceptable salts of such muteins are also an object of the present invention. Such salts can be produced by methods known in the art.

It is believed that the strategy of dissecting beneficial and unwanted TNF-α activities by using compounds specifically binding to one or the other TNF-receptor, such as the hTNF muteins of the present invention, can be used in general in disease states where TNF plays a role.

DNA-sequences coding for hTNF-muteins as hereinbefore described are also an object of the present invention. In addition to their function as an intermediate for obtaining the muteins of the present invention, such sequences (or fragments thereof) can be used in gene therapy, whereby an existing gene can be modified to give beneficial effects. The sequences (or fragments thereof) can also be used as anti-sense DNA for the regulation of gene expression by binding to complementary mRNA sequences.

Such DNA-sequences can be constructed starting from genomic-or cDNA-sequences coding for hTNF as disclosed in the art [see above] using known methods of in vitro mutagenesis [see e.g. Sambrook et al., 1989]. RNA sequences complementary to the DNA sequences of the present invention are also within the scope of the present invention and are of utility e.g. for the preparation of cDNA sequences.

The mutagenesis referred to above can be carried out ad-random in order to obtain a large number of mutants which can then be tested for their desired properties in appropriate assay systems or, in order to mutate defined positions in a given DNA-sequence, by so called site directed mutagenesis [see e.g. Sambrook et al., 1989, 15.51-15.113] or by mutagenesis using the polymerase chain reaction [see e.g. White et al., Trends in Genetics 5, 185-189 (1989)].

One chemical mutagen which is often used for mutagenesis ad-random is sodium bisulfite which converts a cytosine residue into an uracil residue and hence leads to a transition of "C" to "T" (standard abbreviations for nucleotides) [for the method see e.g. Shortle and Nathans, Procd. Nat. Acad. Sci. U.S.A. 75, 2170-2174 (1978) or Pine and Huang, Meth. Enzym. 154, 415-430 (1987)]. This mutagen acts solely on single stranded DNA whereas the expression of the mutated target DNA sequence is achieved with a double stranded plasmid vector. One possibility to avoid the necessity of recloning in mutagenesis and expression vectors is the use of so called "phasmids". These are vectors which, in addition to a plasmid origin of replication, carry also an origin of replication derived from a filamentous phage. Examples of such phasmids are the pMa- and pMc-phasmids as described by Stanssen et al. [Nucleic Acids Res. 17, 4441-4454, (1989)]. Using this expression system one can construct so called "gap-duplex"-structures [see also Kramer et al., Nucl. Acids. Res. 12, 9441-9456 (1984)] where only the TNF-coding sequence (see above) is in a single stranded configuration and therefore accessible for the specific chemical mutagen. "gap-duplexes" to be used in ad-random mutagenesis can be constructed as described for site-specific mutagenesis by Stanssen et al. [see above] with the exception that the (-)strand contains the same active antibiotic resistance gene as the (+)strand. By making use of different restriction sites in the DNA-sequence encoding hTNFα, variation of the width of the gap is possible. Examples of such restriction sites are the C1a1-Sal1 sites (470 nucleotides), BstX1-BstX1 sites (237 nucleotides) or Sty1-Sty1 sites (68 nucleotides). Such gap-duplex-constructs can then be treated with increasing concentrations (up to 4M) of bisulfite, followed by several dialysis steps, as described by Shortle and Nathans (see above). A suitable procaryotic host cell can then be transformed by such phasmid constructs according to methods known in the art and described e.g. by Sambrook et al. (see above). A suitable procaryotic host cell means in this context a host cell deficient in a specific repair function so that an uracil residue is maintained in the DNA during replication and which host cell is capable of expressing the corresponding mutated TNF. Such specific host strains are known in the art, for example for E. coli strains, e.g. E. coli BW 313 [Kunkel, T.A., Procd. Natl. Acad. Sci. USA 82, 488-492 (1985)]. The resulting clones can then be screened for those expressing a desired hTNF mutein by appropriate assay systems. For example each colony can be inoculated in a microtiterplate in a suitable medium containing the relevant antibiotic. The cells may be lysed by addition of lysozyme, followed by sequential freeze-thaw cycles. After precipitation of nucleic acids and centrifugation, the supernatant of each colony can directly be used in appropriate assays as described, e.g., in Example III of the present specification.

If desired, the specific sites of mutation can be determined, for example by restriction fragment analysis [see e.g. Sambrook et al. (see above)]. By determination of the DNA-sequence of such fragments the exact position of the mutation can be determined and if such mutation leads to an amino acid replacement the new amino acid can be derived from the determined DNA-sequence. DNA-sequencing can be performed according to methods known in the art, e.g. by using T7 polymerase on supercoiled DNA with a commercially available sequencing kit (Pharmacia, Uppsala, Sweden).

As already mentioned above, another possibility of mutating a given DNA-sequence is by "site directed mutagenesis". A widely used strategy for such kind of mutagenesis as originally outlined by Hutchinson and Edgell [J. Virol. 8, 181 (1971)] involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single-stranded DNA-sequence wherein the mutation should be introduced [for review see Smith, Annual. Rev. Genet. 19, 423 (1985) and for improved methods see references 2-6 in Stanssen et al. (1989)].

One such preferred method is the one of Stanssen et al. (1989) using "gapped duplex DNA" as originally described by Kramer et al. (1984) [see above and Kramer and Fritz, Methods in Enzymology, (1987), Academic Press, Inc., USA] but using antibiotic resistance genes instead of M13 functional genes for selection of the mutation containing strand in addition with the phasmid-technology as also described by Stanssen et al. (1989) [see above]. An advantage of this method lies also in the capability of performing successive cycles of mutagenesis without the need to transfer the gene to a new mutagenesis vector: second round mutagenesis differs only in the selection to another antibiotic marker (Stranssen et al., see above). As a control site-specific back mutagenesis of the mutant to the wild-type TNF can be used. In addition, the use of an oligonucleotide, creating or destroying a restriction site in the TNF gene, allows to control the mutant not only by hybridization to the oligonucleotide used for site directed mutagenesis but also by the presence or absence of the restriction site. In order to create a set of hTNF muteins wherein at a defined position of their amino acid sequence the wild-type amino acid is replaced by any naturally occurring amino acid a set of oligonucleotides is used with all possible codons at the defined position.

As already mentioned above, another possibility of mutating a given DNA-sequence is the mutagenesis by using the polymerase chain reaction (PCR). The principles of this method are outlined e.g. by White et al. (1989), whereas improved methods are described e.g. in Innis et al. [PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990)].

PCR is an in vitro method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a template DNA. Thereby, PCR is based on the enzymatic amplification of the DNA fragment which is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment between the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. Since the primers are physically incorporated into the amplified product and mismatches between the 5' end of the primer and the template do not significantly affect the efficiency of the amplification, it is possible to alter the amplified sequence thereby introducing the desired mutation into the amplified DNA. By utilizing the thermostable Taq DNA polymerase isolated from the thermophilic bacteria Thermus aquaticus, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers.

Design and synthesis of oligonucleotides can be effected as known in the art and described e.g. in Sambrook et al. (1989) or in one of the references cited above with respect to site directed mutagenesis.

As soon as a DNA-sequence coding for a hTNF-mutein of the present invention has been created, expression can be effected by the phasmid technology as described above or by use of any suitable pro-or eukaryotic expression system well known in the art [see e.g. Sambrook et al., see above].

Expression is effected preferably in prokaryotic cells, e.g., in E. coli, Bacillus subtilis and so on, whereby E. coli, specifically E. coli K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694], WK6 (Stranssens et al. see above) or E. coli SG13009 [Gottesman et al., J. Bacteriol. 148, 265-273 (1981)] are preferred. Expression of the hTNF muteins of the present invention can also be effected in lower or higher eukaryotic cells, like for example yeast cells (like Saccharomyces, Pichia etc.), filamentous fungi (like Aspergillus etc.) or cell lines (like chinese hamster ovary cell lines etc.), whereby expression in yeast cells is preferred [see Sreekrishna et al., Biochem. 28, 4117-4125, (1989); Hitzeman et al., Nature 293, 717-722 (1981); European Patent Application Publication No. 263 311]. Expression of the hTNF muteins of the present invention may occur in such systems either intracellularly, or, after suitable adaption of the gene, extracellularly (see Leemans et al., Gene 85, 99-108, 1989).

Suitable vectors used for expression in E. coli are mentioned e.g. by Sambrook et al. [see above] or by Fiers et al. in "Procd. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris, (Durand et al., eds.), pp. 680-697 (1988)] or and more specifically vectors of the pDS family [Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987); Stüber et al., Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990)] like for example pDS56/RBSII,SphI-TNFα(D143N, A145R) (see Example I) or pDS56/RBSII,SphI-TNFα(mutein) (see Example II), where the term "mutein" represents the TNFα muteins listed in Table 1. Since with these specific pDS56/RBSII-plasmids, due to their specific regulatable promoter/operator elements and ribosomal binding sites, a high level of expression can be achieved, the plasmids can be maintained in E. coli cells only when the activity of the promoter/operator element is repressed by the binding of a lac repressor to the operator. The activity of the promoter can be restored at the desired cell density by addition of IPTG, which inactivates the repressor and clears the promoter. Since most of the E. coli strains do not provide enough repressor molecules to completely repress the function of the promoter sequences present in these high copy number plasmids, such E. coli strains, like E. coli M15 or SG13009, have to be transformed at first with a plasmid, like pREP 4 (see Figures 2a and b), coding for the lac repressor, before being transformed with the specific pDS56/RBSII-plasmids of the invention which can then be stably maintained in the E. coli cells. Beside coding for the lac repressor, pREP4 contains also a region of the plasmid pACYC184 [Chang and Cohen, J. Bacteriol. 134, 1141-1156 (1978)], which contains all information required for replication and stable transmission to daughter cells [for additional information see also "System for high level production in E. coli and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure function analysis" by Stüber et al. in Immunological Methods, Vol. IV, pp 121-152, Lefkovits and Pernis (eds.), Academic Press, New York (1990)].

Transformation of the host cells by vectors as described above may be carried out by any conventional procedure [see for example Sambrook et al. (see above)]. Where the host cell is a prokaryote, such as E. coli for example, competent cells which are capable of DNA uptake are prepared from cells harvested after exponential growth phase and subsequently treated according to the known $CaCl_2$-method. Transformation can also be performed after forming a protoplast of the host cell or by other methods known in the art and described, e.g., in Sambrook et al. (see above). Therefore a vector, especially for expression in a prokaryotic or lower eukaryotic host cell, comprising a DNA-sequence coding for an hTNF mutein as described above, and a host cell, especially a prokaryotic host cell, e.g. E. coli, or a lower eukaryotic host cell, transformed by such a vector are also an object of the present invention.

Usually, the host organisms which contain a desired expression vector are grown under conditions which are optimal for their growth. In case of a procaryotic host at the end of the exponential growth, when the increase in cell number per unit time decreases, the expression of the desired hTNF mutein is induced, i.e. the DNA coding for the desired hTNF mutein is transcribed and the transcribed mRNA is translated. The induction can be carried out by adding an inducer or a derepressor to the growth medium or by altering a physical parameter, e.g. a change in temperature. In the expression vectors used in the preferred embodiments of the present invention, the expression is controlled by the lac repressor. By adding isopropyl-β-D-thiogalactopyranoside (IPTG), the expression control sequence is derepressed and the synthesis of the desired hTNF mutein is thereby induced.

The hTNF muteins of the present invention produced by transformed host cells as stated above can be recovered from the culture medium or after opening the cells and/or extraction by any appropriate method known in protein and peptide chemistry such as, for example, precipitation with ammonium sulfate, dialysis, ultrafiltration, gelfiltration or ion-exchange chromatography, gel electrophoresis, isoelectric focusing, affinity chromatography, like immunoaffinity chromatography, HPLC or the like. Specifically preferred methods are precipitation with ammonium sulfate and/or polyethylenimine, dialysis, affinity chromatography, e.g. on phenyl-agarose, specifically phenyl-sepharose, or ion-exchange chromatography, specifically on a MONO-Q- and/or MONO-S-matrix (Pharmacia, Uppsala, Sweden) or more specifically are those as described by Tavernier et al. [J. Mol. Biol. 211, 493-501 (1990)] and those disclosed in Example IV.

It is therefore also an object of the present invention to provide a process for the preparation of hTNF muteins as specified above which process comprises cultivating a transformed host cell as described above, especially a prokaryotic, e.g. E.coli or eukaryotic host cell in a suitable medium and isolating a mutein from the culture supernatant or the host cell itself, and if desired pegylating said mutein or preparing a pharmaceutically acceptable salt thereof by methods known in the art. The compounds whenever prepared according to such a process are also an object of the present invention.

The hTNF muteins of the present invention are characterized by showing a selective binding affinity for the human p75-TNF-R. Such property can be determined by any assay known in the art measuring binding affinities. For example the binding of TNF itself and of the muteins of the present invention can be

measured using cells in cell culture which express the two types of TNF-receptors to a different degree, like for example Hep-2 cells which exclusivly express the human p55-TNF-R and U937 or HL60 cells which express in addition also the human p75-TNF-R [see Brockhaus et al., Procd. Nat. Acad. Sci. U.S.A. 87, 3127-3131, (1990); Hohmann et al., J. Biol. Chem. 264, 14927-14934, (1989); Loetscher et al. (1990); Dembic et al. (1990)]. Of course binding affinities can also be determined directly by using purified native or recombinant p55-TNF-R and p75-TNF-R as specifically described in the Examples, or by using the corresponding soluble analogs of such receptors.

The term selective binding affinity for the human p75-Tumor-Necrosis-Factor-Receptor" refers in the context of the present invention to a difference in binding affinities to the two types of TNF-receptor. Preferably, with respect to the assay system described in the examples, a mutein of the present invention binds selectively to hp75-TNF-R (desirably to a degree similar to wild-type TNF) but has essentially lost binding to hp55-TNF-R. Desirably, in the context of the assay-system of the Examples, the K_D-value of a specific hTNF mutein of the present invention is at least a factor of 10 or more, more desirably at least a factor of 10^2, larger than for wild-type TNF-α determined by using the in vitro binding assay with recombinant soluble hp55-TNF-R, whereas its K_D-value determined by using the in vitro binding assay in respect of recombinant soluble hp75-TNF-R for the same hTNF mutein desirably differs not by more than a factor of 20 from that of wild-type TNF-α. It should be understood, however, that these specific K_D-values are given for illustration and should not be considered as limiting in any manner.

The hTNF muteins may be administered alone or with one or more additional compounds of the present invention in pharmaceutically acceptable oral, injectable or topical compositions and modes. Administration will be in a dosage such that the amount of the composition in the patient is effective to modify the biological function associated with hTNF mutein function.

Pharmaceutical compositions containing hTNF muteins in association with a compatible pharmaceutically acceptable carrier material are therefore a further object of the present invention. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for enteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavouring agents, preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

The pharmaceutical preparations can be made up in any conventional form including: a) a solid form of oral administration such as tablets, capsules, pills, powders, granules and the like; b) a liquid form for oral administration such as solutions, syrups, suspensions, elixirs and the like; c) preparations for parenteral administration such as sterile solutions, suspensions or emulsions; and d) preparations for topical administrations such as solutions, suspensions, ointments, creams, gels, micronized powders, aerosols and the like. The pharmaceutical preparations may be sterilized and/or may contain adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, salts for varying the osmotic pressure and/or buffers.

Parenteral dosage forms may be infusions or injectable solutions which can be injected intravenously or intramuscularly. These preparations can also contain other medicinally active substances. Additional additives such as preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

Accordingly it is also an object of the present invention to provide a process for the preparation of a pharmaceutical composition which process is characterized in that a compound obtained by a process of the present invention and if desired, additional pharmaceutically active substances are mixed with a non-toxic, inert, therapeutically compatible carrier material and the mixture is brought into a galenical application form.

Furthermore the use of a compound prepared according to a process of the present invention for the preparation of a pharmaceutical composition as described above is also an object of the present invention.

Finally, antibodies can be raised against the hTNF muteins of the present invention. These antibodies can be used in a well-known manner for diagnostic or therapeutic purposes as well as for purification purposes. Such antibodies can be produced by injecting a mammalian or avian animal with a sufficient amount of a vaccine formulation comprising a hTNF mutein of the present invention and a compatible pharmaceutical carrier to elicit the production of antibodies against said hTNF mutein. The appropriate amount of the hTNF mutein which would be required would be known to one of skill in the art or could be determined by routine experimentation. As used in connection with this invention, the term "pharmaceutical carrier" can mean either the standard compositions which are suitable for human administration or the typical adjuvants employed in animal vaccinations.

As pointed out above, TNF is a potent pleiotropic cytokine. Its many different activities such as, for example, the activity of growth factor for immune cells, mediator in inflammation, or inductor of specific genes in endothelium, may be seen in the context of host defense to infection and injury. TNF also exhibits high systemic toxicity; the deleterious effects of bacteriaemia and septic shock or of bacterial meningitis are mediated to a large extent by endogenous cytokines among which TNF has an early and important role. Furthermore, many cells and cell lines are sensitive to a direct cytotoxic activity of TNF. Various systemic effects and cellular toxicity presumably combine in the antitumor activity of TNF seen in animal studies.

These facts form the rational basis for the development of novel therapeutic strategies using the hTNF muteins of the present invention, where in particular the potential to dissect the many different hTNF activities shall be fully exploited to separate unwanted from desired activities by selectively activating only one of the two hTNF receptor types (in contrast to wild-type hTNF which binds and activates both). The potential use of the hTNF muteins of the present invention is not restricted to cancer therapy. Any disease where TNF as host defense factor in bacterial infection [for example Kindler, V. et al., CELL 56, 731-740 (1989); Nakano, Y. et al., J. Immunol. 144, 1935, (1990)] or as mediator in inflammation plays a beneficial role might benefit from a 75kDa TNF receptor type specific drug such as the hTNF muteins of the present invention. Furthermore, TNFα has been shown to have certain catabolic effects on fat cells and on whole animals, and to play a role in cachexia [eg. Beutler, B. and Cerami, (see above); Hotamisligil et al., Science 259, 87 1993] and TNF muteins of the present invention might be used in treating obesity. It also has been shown that TNFα has a neutralising effect on the insulin-stimulated peripheral glucose utilisation rate [Hotamisligil et al., see above]. Such a putative role of TNFα in obesity-linked insulin resistance might be reconciled with its possible role in cachexia by dose-dependent differences in biological effects and distinct roles of the two TNF receptor systems which might be exploited by receptor-type specific agonists in the presence or absence of wild-type TNF-inhibitors. Even disease states characterised by the toxic activities exerted by excessive TNF release such as septic shock or bacterial meningitis might benefit from TNF receptor specific agonists such as the muteins of the present invention above, alone, or in combination with wild-type TNF antagonists.

A concise summary of the emerging role of TNF for novel therapies, where TNF-Receptor type specific agonists selectively triggering only some of the many different TNF activities may be expected to have significant advantages when compared to wild-type TNF, has been published [Tumor Necrosis Factors, The Molecules and their Emerging Role in Medicine, B. Beutler, ed., Raven Press, 1992, ISBN 0-88167-852-X]. It includes the activities of TNF in modulating endothelial cell homeostatic properties and neutrophil adhesion, tissue ischemia and reperfusion injury, on osteoblasts and osteoclasts in bone resorption, as growth factor on many cells in general and in hematopoiesis, as well as in metabolic and nutritional effects. TNF as a growth/differentiation factor in the generation of lymphokine-activated killer (LAK) cells appears to contribute to the antitumor activities of TNF. Accordingly the use of the hTNF-muteins of the present invention or of pharmaceutically acceptable salts thereof is also an object of the present invention.

All these activities may be enhanced or modulated in combination with other recombinant cytokines such as, for example, interferongamma.

The present invention will now be described, by way of example only, with resepct to the accompanying drawing,

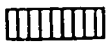
Abbreviations and symbols used are:

B, E, H, S, Xb and X which indicate cleavage sites for restriction enzymes BglI, EcoRI, HindIII, SalI, XbaI and XhoI, respectively.

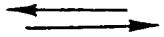
represents the regulatable promoter/operator element N25OPSN25OP29,

represents the synthetic ribosomal binding site RBSII,SphI,

represents genes for TNFα (TNFα), β-lactamase (bla), chloramphenicol acetyltransferase (cat), lac repressor (lacI) and neomycin phosphotransferase (neo),



represents transcriptional terminators t_0 of phage lambda (t_0) and T1 of the E. coli rrnB operon (T1)



represents the replication regions of plasmids pBR322 and pREP4 (repl.),

represents the coding region under control of N250PSN250P29 and RBSII,SphI.

Figure 1a is a schematic drawing of the plasmid pDS56/RBSII,SphI-TNFα.

Figure 1b displays the complete nucleotide sequence of plasmid pDS56/RBSII,SphI-TNFα (SEQ ID No. 1). In this sequence, the recognition sequences of the restriction enzymes depicted in Figure 1a are indicated. The amino acid sequence shown represents in the three letter code the sequence of the mature human TNFα (157 amino acids; SEQ ID No. 1 and 2).

Figure 2a is a schematic drawing of the plasmid pREP4.

Figure 2b displays the complete nucleotide sequence of plasmid pREP4 (SEQ ID No. 3). In this sequence, the recognition sequences of the restriction enzymes depicted in Figure 2a are indicated (see also Figures 2b1-2b3 of EP 486 908).

Figure 3 outlines the preparation of an EcoRI-HindIII fragment encoding the TNFα mutein TNFα - (D143N,A145R).

Figure 4 illustrates the Competitive binding of Human Wild-type TNFα and D143N, A145R and D143N-A145R Muteins to Human TNFR-p75 and TNFR-p55. 96 well microtiter plates coated with recombinant human TNFR-p75-hγ3 fusion protein (upper panel) and recombinant human TNFR-p55-hγ3 fusion protein (lower panel) were incubated with radiolabelled human TNFα in the presence of different concentrations of unlabelled wild-type TNFα, D143N, A145R or D143N-A145R muteins. After three hours at room temperature bound radioactivity was counted in a γ-counter.

Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively.

Example I

Preparation of TNFα(D143N-A145R)

Plasmid pDS56/RBSII,SphI-TNFα

The human TNFα expression plasmid pDS56/RBSII,SphI-TNFα (see Figure 1) was the source of the TNFα gene for preparation of the various TNFα muteins of this invention. The transformed E. coli strain M15 [pREP4;pDS56/RBSII,SphI-TNFα] has been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, BRD, at September 8, 1991, under the accession number DSM 6713.

Mutagenesis of the TNFα gene using PCR

Two PCR reactions were performed with plasmid pDS56/RBSII,SphI-TNFα (Figure 1) as the template DNA using a Perkin-Elmer Cetus GeneAmp™ DNA Amplification Reagent Kit with AmpliTaq™ Recombinant Taq DNA Polymerase [see Figure 3].

Reaction I was performed with primers 17/F [5'-GGCGTATCACGAGGCCCTTTCG-3' (SEQ ID No. 4); primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,SphI-TNFα] and 46/M12 [5'-GCGAAAGTTGAGATAGTCGGGCCGATTG-3' (SEQ ID No. 5); primer 46/M12 comprises nucleotides which

are complementary to nucleotides 552-525 of plasmid pDS56/RBSII,SphI-TNFα, the mutated bases are underlined].

Reaction II was performed with primers 29/MR2 [5'-GAGTCTGGGCAGGTCTACTTTG-3' (SEQ ID No. 6); primer 29/MR1 comprises nucleotides 553-574 of plasmid pDS56/RBSII,SphI-TNFα] and 17-O [5'-CATTAC TGGATCTATCAACAGG-3' (SEQ ID NO. 7); primer 17-O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,SphI-TNFα].

In a typical experiment, 10 µl template DNA (10 ng), 5 µl each of the two primers (100 pmoles each), 16 µl dNTP's mix (1.25 mM of dATP, dGTP, dCTP, and dTTP), 10 µl 10x reaction buffer (100 mM Tris-HCl pH8.3, 500 mM KCL, 15 mM $MgCl_2$ and 0.1 % gelatin), 1 µl (5 units) AmpliTaq™ DNA polymerase and 53 µl H_2O were mixed in an Eppendorf tube and overlaid with 80 ml mineral oil (Perkin-Elmer Cetus). The tubes were transferred to a DNA thermal cycler (TRIO-Thermoblock. Biometra) and kept for 1 min at 94°C, before 35 cycles of melting the DNA (1 min at 94°C), annealing the primers (1 min at 50°C), and extending the primers (3 min at 72°C) were performed. After additional 2 min at 72°C, the reactions were cooled to room temperature and extracted with chloroform. The DNA present in the aqueous phase was precipitated with ethanol and subjected to electrophoresis in a 6 % polyacrylamide gel [Sambrook et al., 1989]. After staining of the DNA with ethidium bromide, fragments I and II (see Figure 3) were isolated from the gel and purified [Sambrook et al., 1989].

Preparation of a DNA fragment encoding TNFα (D143N-A145R)

Fragments I and II were enzymatically phosphorylated, before they were ligated with each other [Sambrook et al., 1989]. After heat-inactivation of the ligase and digestion with restriction enzymes EcoRI and HindIII, the DNA was subjected to electrophoresis in a 6 % polyacrylamide gel. After staining of the DNA with ethidium bromide, the EcoRI-HindIII fragment A [see Figure 3] was isolated from the gel and purified [see above].

Preparation of a plasmid encoding TNFα (D143N-A145R)

The EcoRI-HindIII fragment A was inserted according to standard methods [Sambrook et al., 1989] into the EcoRI-HindIII opened plasmid pDS56/RBSII,SphI-TNFα generating the plasmid pDS56/RBSII,SphI-TNFα(D143N-A145R). Plasmid DNA was prepared [Birnboim et al., 1979] and the identity of the coding region for the TNFα mutein was confirmed by sequencing the double-stranded DNA [Sambrook et al., 1989].

Production of TNFα (D143N-A145R)

Plasmid pDS56/RBSII,SphI-TNFα(D143A-A145R) was transformed into E. coli M15 cells containing already plasmid pREP4 by standard methods [see above]. Transformed cells were grown at 37°C in LB medium [Sambrook et al., 1989] containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2 mM. After additional 2.5 to 5 h at 37°C the cells were harvested by centrifugation.

Example II

Preparation of additional TNFα muteins

The additional TNFα muteins listed in Table I were prepared following the procedure described in detail in Example I for the preparation of TNFα (D143N-A145R). The resulting expression plasmids, which are analogous to plasmid pDS56/RBSII,SphI-TNFα (D143N-A145R),were given the name pDS56/RBSII,SphI-TNFα (mutein), where the term 'mutein' represents the TNFα muteins listed in Table 1. These plasmids contain coding regions for the TNFα muteins, in which codons present in plasmid pDS56/RBSII,SphI-TNFα are replaced by codons encoding the said muteins (see Table 1).

Example III

Analysis of Receptor Type-Specific Binding Activity of Human TNFα Muteins in E. coli Lysates

Preparation of E. coli Lysates

10 ml suspensions of E. coli cells transformed and induced as described in Examples I and II were centrifuged at 4'000 rpm for 10 min and resuspended in 0.9 ml of lysis buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 200 units/ml aprotinine and 0.1 mg/ml lysozyme). After 20 min incubation at room temperature 50 µl of 1 M $MgCl_2$, 20 µl of 5 mg/ml DNaseI, 50 µl of 5 M NaCl and 50 µl of 10% NP-40 were added and the mixture was further incubated at room temperature for 15 min. 0.5 ml of the lysate clarified by centrifugation at 13'000 rpm for 5 min was subjected to ammonium sulfate precipitation (25% - 70% cut). The 70% ammonium sulfat pellet was dissolved in 0.2 ml PBS and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm presence and approximate amount of the recombinant proteins.

Solid Phase Radioligand Competition Binding Assay

96 well microtiter plates were coated with recombinant human TNFR-p75-hγ3 and TNFR-p55-hγ3 fusion proteins (extracellular portion of the receptor fused to the Fc part of human IgG3) at a concentration of 0.3 µg/ml and 0.1 µg/ml, respectively, in phosphate buffered saline (PBS, 100 µl/well, overnight at 4°C) [Loetscher, H. et al. J. Biol. Chem. 266, 18324 - 18329 (1991); Lesslauer, W. et al. Eur. J. Immunol. 21, 2883 - 2886 (1991)]. After blocking with blocking buffer (50 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% NaN3, 1% defatted milk powder) the microtiter plate was washed with PBS and incubated in blocking buffer containing 0.1% defatted milk powder with 10 ng/ml human wild-type ^{125}I-TNFα and various dilutions of E. coli lysates ranging from 10^{-2} to 10^{-7} (10-fold serial dilutions). TNFα was labelled by the Iodogen method (Pierce Chemical Company) to a specific activity of about 10-30 µCi/µg. The volume was 100 µl/well and each lysate dilution was assayed in duplicate or triplicate. After three hours at room temperature the wells were thoroughly washed with PBS and counted in a γ-counter. The results are given in Table 2 for lysates comprising the muteins indicated therein.

Example IV

Purification of Human TNFα Muteins

One liter overnight cultures of E. coli cells transformed and induced as described in the Examples I and II were collected by centrifugation and resuspended in 20 ml 50 mM Tris pH 7.2, 200 mM KCl, 50 mM $MgCl_2$, 5% glycerol. The cells were disrupted in a French press at a pressure of 20'000 psi or by sonication in a Branson Sonifier (Model 450, 2 x 2min at maximal output, on ice). After clarification by centrifugation (70'000 x g, 30 min, 4°C) the samples were dialyzed against 20 mM Tris-HCl pH 9.0 overnight at 4°C and applied to a Q-Sepharose column (Pharmacia, 2.6 x 15 cm) equilibrated in the same buffer. Proteins were eluted with a linear NaCl gradient (0 to 400 mM in 20 mM Tris pH 9.0) at a flow rate of 1 ml/min. 5 ml fractions were collected and analyzed for the presence of TNFα muteins by SDS-PAGE. Positive fractions were pooled, dialyzed against 20 mM 2-morpholino-ethanesulfonic acid (MES) pH 6.0 and applied to a MonoS column (HR 5/5, LKB-Pharmacia) equilibrated in 20 mM MES pH 6.0. Proteins were eluted with a linear NaCl gradient (0 to 400 mM in 20 mM MES pH 6.0) at a flow rate of 0.5 ml/min. The various TNFα muteins eluted as electrophoretically pure proteins between 250 mM and 350 mM NaCl. After dialysis against PBS the protein concentration was determined by the BCA Protein Assay (Pierce Chemical Company) using wild-type human TNFα as a standard or by absorbance measurements at 280 nm.

Example V

Competitive Binding of Purified Human Wild-type TNFα and Muteins to Recombinant Human TNFR-p75 and TNFR-p55

For the competitive binding assay using purified muteins microtiter plates were coated with recombinant human TNFR-p75-hγ3 and TNFR-p55-hγ3 fusion proteins as described in Example III. After blocking with blocking buffer (50 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% NaN3, 1% defatted milk powder) the microtiter plate was washed with PBS and incubated in blocking buffer containing 0.1% defatted milk powder with 10 ng/ml human wild-type ^{125}I-TNFα and various concentrations of unlabelled wild-type TNFα or muteins ranging from 10^{2} to 10^{-5} µg/ml (10-fold serial dilutions). TNFα was labelled by the Iodogen method (Pierce Chemical Company) to a specific activity of about 10-30 µCi/µg. The volume was 100

µl/well and each concentration was assayed in duplicate or triplicate. After three hours at room temperature the wells were thoroughly washed with PBS and counted in a γ-counter.
The results are given in Table 3 and illustrated in Fig. 4 for the muteins indicated therein.

Table 1

Codons used to encode the new amino acids present in the muteins

Mutein	New Codon
N19D	GAC
Q21S	TCT
L29S[a]	TCC
L29S-R32W	TCC-TGG
L29S-R32W-S86T	TCC-TGG-ACC
L29S-S86T	TCC-ACC
N30T	ACC
R31E	GAG
R31K	AAG
R31N-R32T	AAC-ACT
R31N-R32T-N34S	AAC-ACT-AGT
R31N-R32T-S86T	AAC-ACT-ACC
R31E-S86T	GAG-ACC
R32W[a]	TGG
R32W-S86T	TGG-ACC
A33D	GAC

A33T	ACC
N34R	CGT
N34D	GAC
N34C	TGT
N34Q	CAA
N34E	GAA
N34G	GGT
N34H	CAC
N34I	ATT
N34M	ATG
N34F	TTT
N34P	CCT
N34T	ACT
N34Y	TAT
N34Y	TAC
N34V	GTT

K65A	GCA
K65W	TGG
Q67K	AAA
Q67T	ACA
Q67Y	TAC
H73Q	CAA
H73T	ACT
L75R	CGT
L75H	CAC

L75W	TGG
S86D	GAC
S86T	ACC
Y87Q	CAG
Y87Q-Q88Δ	CAG-
Y87E	GAA
Y87G	GGT
Y87L	CTG
Y87K	AAA
Y87F	TTC
Y87T	ACC
Y87T-E104G	ACC-GGG
N92R	CGT
I97K	AAG
I97Y	TAC
S99A	GCA
S99Y	TAC
Y115W	TGG
D143N	AAC
D143E	GAA
D143F	TTC
D143W	TGG

D143Y	TAC
D143V	GTC
D143V-F144L-A145S	GTC-CTG-TCC
D143N-A145R	AAC-CGC
D143V-A145S	GTC-TCC
F144R	CGT
F144D	GAT
F144G	GGT
F144L	TTG
F144W	TGG
F144Y	TAC
A145R	CGC
A145D	GAT
A145G	GGT
A145H	CAC
A145K	AAA
A145F	TTT
A145S	TCC
A145T	ACA
A145W	TGG
A145Y	TAC
A145V	GTT
E146R	CGT
S147N	AAC
S147L	CTG

a the L29S and R32W muteins have been constructed in the laboratory of Dr. W. Fiers, University of Ghent (see also EP 486 908).

Table 2

Binding of human TNFα Muteins to TNFR-p55 and TNFR-p75

Mutein	Dilution of E. coli Lysate for 50% Inhibition of ^{125}I-TNFα Binding, ID50 a)		ID50 TNFR-p55 b) / ID50 TNFR-p75
	TNFR-p55	TNFR-p75	
	- *fold*	- *fold*	
wildtype c)	14'260	14'140	1
N19D	5'000	5'000	1
Q21S	2'500	2'500	1
L29S c) e)	2'980	<100	>29.8
L29S-R32W	5'000	<<100	>>50
L29S-R32W-S86T	2'500	<<100	>>25
L29S-S86T	200	<<100	>>2
N30T	2'860	2'500	1.1

R31E [c]	3'470	180	19.3
R31K	3'330	3'330	1
R31N-R32T [c]	3'260	<100	>32.6
R31N-R32T-N34S	<<100	<<100	1
R31N-R32T-S86T	500	<100	>5
R31E-S86T	2'000	<<100	>>20
R32W [c] [e]	8'780	<100	>87.8
R32W-S86T	3'330	<<100	>>33.3
A33D	<100	<<100	>1
A33T	1'110	1'250	0.9
N34R [d]	<100	<<100	>1
N34D	250	<100	>2.5
N34C	250	<100	>2.5
N34Q	<100	<<100	>1
N34E	330	<<100	>>3.3
N34G	330	<100	>3.3
N34H	670	<100	>6.7
N34I [d]	200	<100	>2
N34M [d]	<100	<<100	>1
N34F [d]	100	<100	>1
N34P	<100	<<100	>1

N34T	1'000	<100	>10
N34Y *d)*	<100	<<100	>1
N34Y *d)*	<100	<<100	>1
N34V *d)*	<100	<<100	>1
K65A	20'000	33'330	0.6
K65W *d)*	500	3'330	0.2
Q67K	25'000	50'000	0.5
Q67T	25'000	33'330	0.75
Q67Y	20'000	33'330	0.6
H73Q	10'000	10'000	1
H73T	2'000	2'000	1
L75R	<100	<100	1
L75H	1'670	2'500	0.7
L75W	220	330	0.7
S86D	6'670	1'000	6.7
S86T	10'000	<100	>100
Y87Q	<<100	<<100	1
Y87Q-Q88Δ	<<100	<<100	1
Y87E	<100	<<100	>1

Y87G	<<100	<<100	1
Y87L	<<100	<<100	1
Y87K	<<100	<<100	1
Y87F	200	<100	>2
Y87T	<<100	<<100	1
Y87T-E104G	<100	<100	1
N92R	5'000	1'250	4
I97K	143	<100	>1.4
I97Y	2'500	330	7.6
S99A	6'670	6'670	1
S99Y	<100	<100	1
Y115W	2'220	2'220	1
D143N c)	<<100	330	<<0.3
D143E	<100	330	<0.3
D143F	<<100	250	<<0.4
D143W	<<100	100	<<1
D143Y c)	<<100	1'330	<<0.08
D143V	<<100	<100	<1
D143V-F144L-A145S	<<100	<100	<1

D143N-A145R *d)*	<<100	125	<<0.8
D143V-A145S *c)*	<<100	200	<<0.5
F144R	2'500	330	7.6
F144D	5'000	330	15.2
F144G	2'500	2'000	1.2
F144L	5'000	5'000	1
F144W	400	180	2.2
F144Y	2'860	2'860	1
A145R	<100	3'330	<0.03
A145D	5'000	6'670	0.7
A145G	2'500	6'670	0.4
A145H	330	1'670	0.2
A145K	<100	1'820	<0.05
A145F *c)*	240	6'000	0.04
A145S	14'290	25'000	0.6
A145T	5'000	6'670	0.7
A145W *d)*	<<100	<100	<1
A145Y	1'670	11'110	0.1
A145V	1'000	2'000	0.5
E146R	6'670	<100	>67
S147N	10'000	10'000	1

S147L	2'000	3'330	0.6

Human wildtype TNFα and muteins were expressed in E. coli and extracted by lysis of the bacteria. Selective receptor binding activity of extracted wildtype and mutant TNFα was measured in a solid phase radioligand binding assay. Different dilutions of the E. coli lysates ranging from 10^{-2} to 10^{-7} (10-fold serial dilutions) were tested for competitive binding inhibition of human wildtype ^{125}I-TNFα to immobilized human TNFR-p75 and TNFR-p55. ID50's (dilution for 50% inhibition) were determined by plotting binding inhibition versus dilution of the lysate. Since the concentration of the recombinant proteins in the lysates varied between 0.05 and 1 mg/ml as estimated from SDS-PAGE analysis, the absolute ID50 values should not be considered as relevant. Receptor selectivity is indicated by directly comparing the ID50 values of a particular mutein for TNFR-p75 and TNFR-p55.

a) "<" indicates a value less than that of the figure given (here there was measurable inhibition of ^{125}I-TNFα binding but without reaching 50% inhibition at the lowest dilution tested of 1:100).

"<<" indicates a value considerably less than that of the figure given (here there was no measurable inhibition at the lowest dilution tested of 1:100).

b) ratio = 1, no receptor selectivity;

ratio > 1, TNFR-p55 selectivity;

ratio < 1, TNFR-p75 selectivity;

Muteins of the present invention should have an

ID50 TNFR-p55

ID50 TNFR-p75

value of less than 1. This can be less than 0.5, but is preferably less than or equal to 0.2 (see the muteins of claim 5) and more preferably is less than or equal to 0.1 (see the muteins of claim 6). Where the symbols "<" or "<<" are used in Table 1, the muteins concerned shall, optionally, be considered to be within these ranges.

c) for these muteins at least three different lysates have been prepared and assayed; the average ID50's are listed.

d) these muteins were only partially soluble under the conditions used to prepare the E. coli lysate (although it should be noted that different purification methods could be used resulting in different solubilities); the concentration of soluble mutein in these lysates was estimated by SDS-PAGE analysis to be less than 0.05 mg/ml.

e) the L29S and R32W muteins have been constructed in the laboratory of Dr. W. Fiers, University of Ghent (see also EP 486 908).

Table 3

Binding of Selected Human TNFα Muteins to Human TNFR-p75 and TNFR-p55

Mutein	Mutein Concentration for 50% Inhibition of ^{125}I-TNFα Binding, IC50 *a)*		Decrease in Binding Affinity with Respect to Wildtype *b)*	
	TNFR-p55	TNFR-p75	TNFR-p55	TNFR-p75
	ng/ml	*ng/ml*	*-fold*	*-fold*
D143N	>100'000	300	>2'500	6.7
D143Y	>100'000	350	>6'660	17.5
A145F	500	30	33	1.5
A145R	100'000	35	2'500	0.8
A145W	10'000	100	250	2.5
D143N-A145R	>>100'000	300	>>2'500	6.7

Muteins with preferential binding to human TNFR-p75 were selected (see Table 2) and purified to apparent homogeneity by sequential ion exchange

chromatography. Selective receptor binding activity of the purified muteins was measured in a solid phase radioligand binding assay. Different mutein concentrations ranging from 10^2 to 10^{-5} µg/ml (10-fold serial dilutions) were tested for competitive binding inhibition of human wildtype ^{125}I-TNFα (10 ng/ml) to immobilized human TNFR-p75 and TNFR-p55. IC50's (concentration for 50% inhibition) were determined by plotting binding inhibition versus concentration (illustrated in Figure 1).

a) >, indicates measurable binding competition but without reaching 50% at 100 µg/ml;

>>, indicates no measurable binding competition at the highest concentration tested (100 µg/ml).

b) The decrease in affinity has been calculated by dividing the IC50 values obtained for the muteins by the IC50 values obtained for wildtype TNFα. The IC50 value for wildtype TNFα has been determined in each individual set of experiments and was found to vary between 15 to 45 ng/ml depending on the lot of radioiodinated TNFα.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: F. HOFFMANN-LA ROCHE AG
(B) STREET: Grenzacherstrasse 124
(C) CITY: Basle
(D) STATE: BS
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): CH-4002
(G) TELEPHONE: 061 - 688 25 05
(H) TELEFAX: 061 - 688 13 95
(I) TELEX: 962292/965542 hlr ch

(ii) TITLE OF INVENTION: Tumor Necrosis Factor Muteins

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 93810224.1
(B) FILING DATE: 29-MAR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3977 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plasmid pDS56/RBSII,SphI-TNFalpha

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 118..591

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG AGGAGAAATT AAGCATG 117

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            20                  25                  30

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Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
        35                  40                  45

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Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
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Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65                  70                  75                  80

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Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
                85                  90                  95

ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC AAG        453
Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
            100                 105                 110

CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG AAG        501
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
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GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC TTT        549
Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
    130                 135                 140

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Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145                 150                 155

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ACGCTCGGTT GCCGCCGGGC GTTTTTTATT GGTGAGAATC CAAGCTAGCT TGGCGAGATT      838

TTCAGGAGCT AAGGAAGCTA AAATGGAGAA AAAAATCACT GGATATACCA CCGTTGATAT      898

ATCCCAATGG CATCGTAAAG AACATTTTGA GGCATTTCAG TCAGTTGCTC AATGTACCTA      958

TAACCAGACC GTTCAGCTGG ATATTACGGC CTTTTTAAAG ACCGTAAAGA AAAATAAGCA     1018

CAAGTTTTAT CCGGCCTTTA TTCACATTCT TGCCCGCCTG ATGAATGCTC ATCCGGAATT     1078

TCGTATGGCA ATGAAAGACG GTGAGCTGGT GATATGGGAT AGTGTTCACC CTTGTTACAC     1138

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CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT    3838

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GAGGCCCTTT CGTCTTCAC                                                 3977
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1               5                  10                  15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
            20                  25                  30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
        35                  40                  45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
    50                  55                  60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
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65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
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Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
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Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
115 120 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
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Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3740 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plasmid pREP4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAGCTTCACG CTGCCGCAAG CACTCAGGGC GCAAGGGCTG CTAAAGGAAG CGGAACACGT 60

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GAGTTAGTCT TGAAGTCATG CGCCGGTTAA GGCTAAACTG AAAGGACAAG TTTTGGTGAC 3480

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AAACCGCCCT GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGCG CAGACCAAAA 3600

CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTC AGTGCAATTT 3660

ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT ATAAGTTGTT AATTCTCATG 3720

TTTGACAGCT TATCATCGAT 3740

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 17/F

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCGTATCAC GAGGCCCTTT CG 22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 46/M12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCGAAAGTTG AGATAGTCGG GCCGATTG 28

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 29/MR2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GAGTCTGGGC AGGTCTACTT TG 22

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 17/0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CATTACTGGA TCTATCAACA GG 22

Claims

1. A human TNFα mutein having higher binding affinity for human p75-TNF receptor than for human P55 TNF receptor or a pharmaceutically acceptable salt thereof.

2. A compound according to claim 1 comprising at least one different amino acid to wild-type human TNFα at a position corresponding to position 33, 65, 67, 75, 143, 145 and/or 147 of wild-type human TNFα.

3. A compound according to claim 2, wherein the mutein comprises at least one different amino acid at a position corresponding to position 143 and/or 145 of wild-type human TNFα.

4. A compound according to claim 2, wherein the mutein comprises at least one of the following amino acid changes at a position corresponding to the position indicated for the wild-type sequence:
A33T
K65A
K65W
Q67K
Q67T
Q67Y
L75H
L75W
D143N
D143E
D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145D
A145G
A145H
A145K
A145F
A145S
A145T
A145W
A145Y
A145V
E146R
S147L;

5. A compound according to claim 4, wherein the mutein comprises at least one of the following amino acid changes at a position corresponding to the position indicated for the wild-type sequence:

K65W
D143N
D143E
D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145H
A145K
A145F
A145W
A145Y

6. A compound according to claim 5, wherein the mutein comprises at least one of the following amino acid changes at a position corresponding to the position indicated for the wild-type sequence:

D143N
D143E
D143F
D143W
D143Y
D143V
D143V - F144L - A145S
D143N - A145R
D143V - A145S
A145R
A145K
A145F
A145W
A145Y

7. A compound according to any preceding claim, wherein the mutein comprises at least one of the following amino acid changes at a position corresponding to the position indicated for the wild-type sequence:

D143N
D143Y
A145F
A145R
A145W
D143N - A145R

8. A compound as claimed in any one of claims 1-7 in pegylated form.

9. A DNA sequence coding for a mutein according to any of claims 1 to 7.

10. A vector comprising a DNA sequence according to claim 9.

11. A host cell comprising a vector according to claim 10.

12. An RNA sequence complementary to a DNA sequence according to claim 10.

13. A compound according to any of claims 1 to 8 for use in the treatment of the human or animal body by surgery, or therapy or for use in a diagnostic method.

14. A process for the preparation of a compound as claimed in any of claims 1 to 8 which comprises cultivating a host cell as claimed in claim 11 in a suitable medium, and then purifying the mutein and optionally pegylating the so purified mutein or preparing a salt thereof by methods known in the art.

15. A pharmaceutical composition comprising a compound according to any of claims 1-8, in combination with a pharmaceutical acceptable carrier.

16. The use of a compound as claimed in any one of claims 1-8 for the treatment of the human or animal body by surgery or therapy or in a diagnostic method.

FIG. 1A

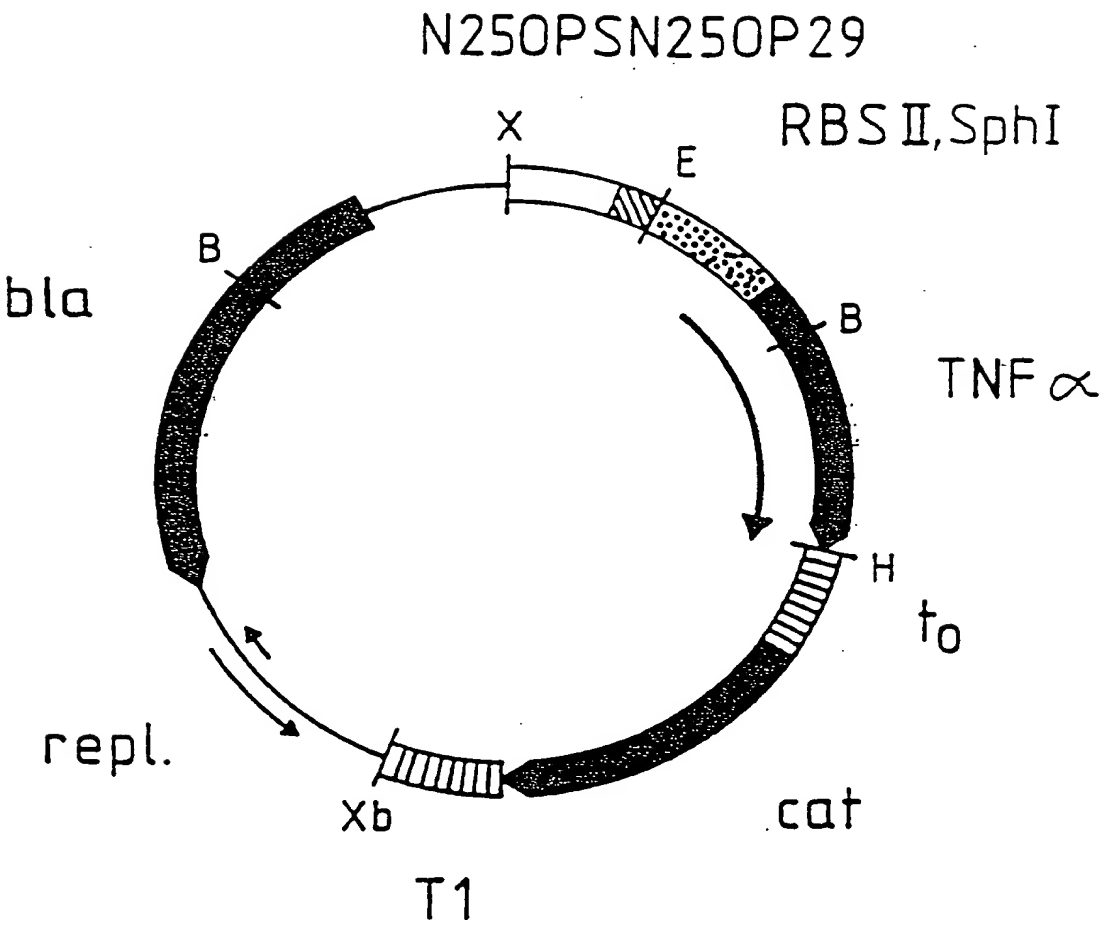

FIG. 1B/1

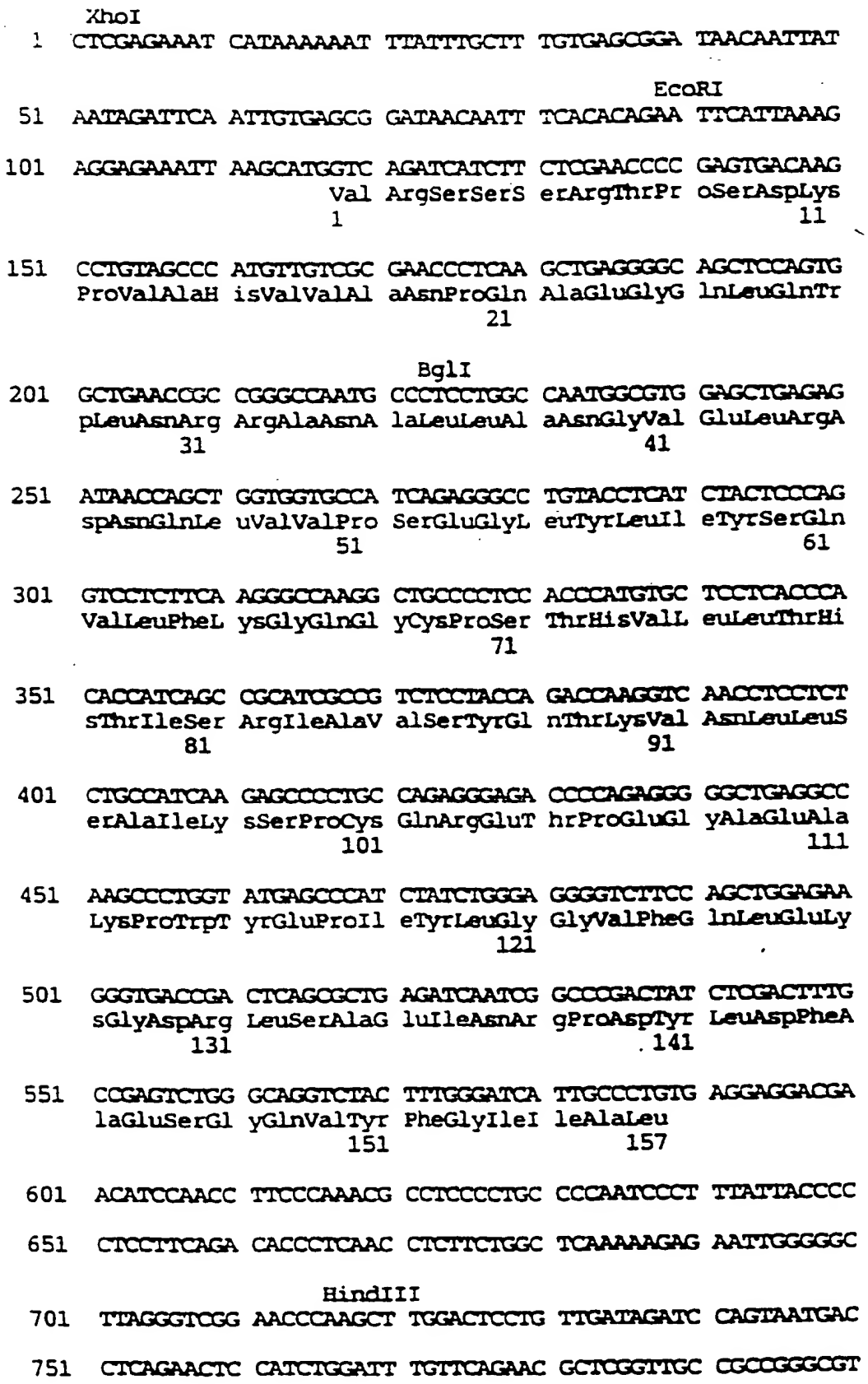
XhoI
1 CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
101 AGGAGAAATT AAGCATGGTC AGATCATCTT CTCGAACCCC GAGTGACAAG
Val ArgSerSerS erArgThrPr oSerAspLys
1 11
151 CCTGTAGCCC ATGTTGTCGC GAACCCTCAA GCTGAGGGGC AGCTCCAGTG
ProValAlaH isValValAl aAsnProGln AlaGluGlyG lnLeuGlnTr
21
BglI
201 GCTGAACCGC CGGGCCAATG CCCTCCTGGC CAATGGCGTG GAGCTGAGAG
pLeuAsnArg ArgAlaAsnA laLeuLeuAl aAsnGlyVal GluLeuArgA
31 41
251 ATAACCAGCT GGTGGTGCCA TCAGAGGGCC TGTACCTCAT CTACTCCCAG
spAsnGlnLe uValValPro SerGluGlyL euTyrLeuIl eTyrSerGln
51 61
301 GTCCTCTTCA AGGGCCAAGG CTGCCCCTCC ACCCATGTGC TCCTCACCCA
ValLeuPheL ysGlyGlnGl yCysProSer ThrHisValL euLeuThrHi
71
351 CACCATCAGC CGCATCGCCG TCTCCTACCA GACCAAGGTC AACCTCCTCT
sThrIleSer ArgIleAlaV alSerTyrGl nThrLysVal AsnLeuLeuS
81 91
401 CTGCCATCAA GAGCCCCTGC CAGAGGGAGA CCCCAGAGGG GGCTGAGGCC
erAlaIleLy sSerProCys GlnArgGluT hrProGluGl yAlaGluAla
101 111
451 AAGCCCTGGT ATGAGCCCAT CTATCTGGGA GGGGTCTTCC AGCTGGAGAA
LysProTrpT yrGluProIl eTyrLeuGly GlyValPheG lnLeuGluLy
121
501 GGGTGACCGA CTCAGCGCTG AGATCAATCG GCCCGACTAT CTCGACTTTG
sGlyAspArg LeuSerAlaG luIleAsnAr gProAspTyr LeuAspPheA
131 141
551 CCGAGTCTGG GCAGGTCTAC TTTGGGATCA TTGCCCTGTG AGGAGGACGA
laGluSerGl yGlnValTyr PheGlyIleI leAlaLeu
151 157
601 ACATCCAACC TTCCCAAACG CCTCCCCTGC CCCAATCCCT TTATTACCCC
651 CTCCTTCAGA CACCCTCAAC CTCTTCTGGC TCAAAAAGAG AATTGGGGGC
HindIII
701 TTAGGGTCGG AACCCAAGCT TGGACTCCTG TTGATAGATC CAGTAATGAC
751 CTCAGAACTC CATCTGGATT TGTTCAGAAC GCTCGGTTGC CGCCGGGCGT

FIG. 1B/2

```
 801  TTTTTATTGG TGAGAATCCA AGCTAGCTTG GCGAGATTTT CAGGAGCTAA
 851  GGAAGCTAAA ATGGAGAAAA AAATCACTGG ATATACCACC GTTGATATAT
 901  CCCAATGGCA TCGTAAAGAA CATTTTGAGG CATTTCAGTC AGTTGCTCAA
 951  TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC
1001  CGTAAAGAAA AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG
1051  CCCGCCTGAT GAATGCTCAT CCGGAATTTC GTATGGCAAT GAAAGACGGT
1101  GAGCTGGTGA TATGGGATAG TGTTCACCCT TGTTACACCG TTTTCCATGA
1151  GCAAACTGAA ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC
1201  GGCAGTTTCT ACACATATAT TCGCAAGATG TGGCGTGTTA CGGTGAAAAC
1251  CTGGCCTATT TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC
1301  CAATCCCTGG GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG
1351  ACAACTTCTT CGCCCCCGTT TTCACCATGG GCAAATATTA TACGCAAGGC
1401  GACAAGGTGC TGATGCCGCT GGCGATTCAG GTTCATCATG CCGTCTGTGA
1451  TGGCTTCCAT GTCGGCAGAA TGCTTAATGA ATTACAACAG TACTGCGATG
1501  AGTGGCAGGG CGGGGCGTAA TTTTTTTAAG GCAGTTATTG GTGCCCTTAA
1551  ACGCCTGGGG TAATGACTCT CTAGCTTGAG GCATCAAATA AAACGAAAGG
1601  CTCAGTCGAA AGACTGGGCC TTTCGTTTTA TCTGTTGTTT GTCGGTGAAC

                                          XbaI
1651  GCTCTCCTGA GTAGGACAAA TCCGCCGCTC TAGAGCTGCC TCGCGCGTTT
1701  CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA
1751  CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG
1801  TCAGCGGGTG TTGGCGGGTG TCGGGGCGCA GCCATGACCC AGTCACGTAG
1851  CGATAGCGGA GTGTATACTG GCTTAACTAT GCGGCATCAG AGCAGATTGT
1901  ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA TGCGTAAGGA
1951  GAAAATACCG CATCAGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG
2001  CGCTCGGTCT GTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT
2051  AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG
2101  CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG
```

FIG. 1B/3

```
2151  TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC
2201  AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC
2251  CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC
2301  GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG
2351  CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG
2401  GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT
2451  AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC
2501  AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA
2551  CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA
2601  TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG
2651  TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG
2701  TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT
2751  TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA
2801  AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT
2851  TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT
2901  TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT
2951  CTGTCTATTT CGTTCATCCA TAGCTGCCTG ACTCCCCGTC GTGTAGATAA
3001  CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG
3051  CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC

          BglI
3101  CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC
3151  AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT
3201  AGTTTGCGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC
3251  GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG
3301  TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT
3351  CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT
3401  GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT
3451  CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG
```

FIG. 1B/4

3501 CGACCGAGTT GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA

3551 TAGCAGAACT TTAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA

3601 AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT

3651 CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG

3701 GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA

3751 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA TTATTGAAGC

3801 ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA

3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC

3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG

3951 CGTATCACGA GGCCCTTTCG TCTTCAC

FIG. 2A

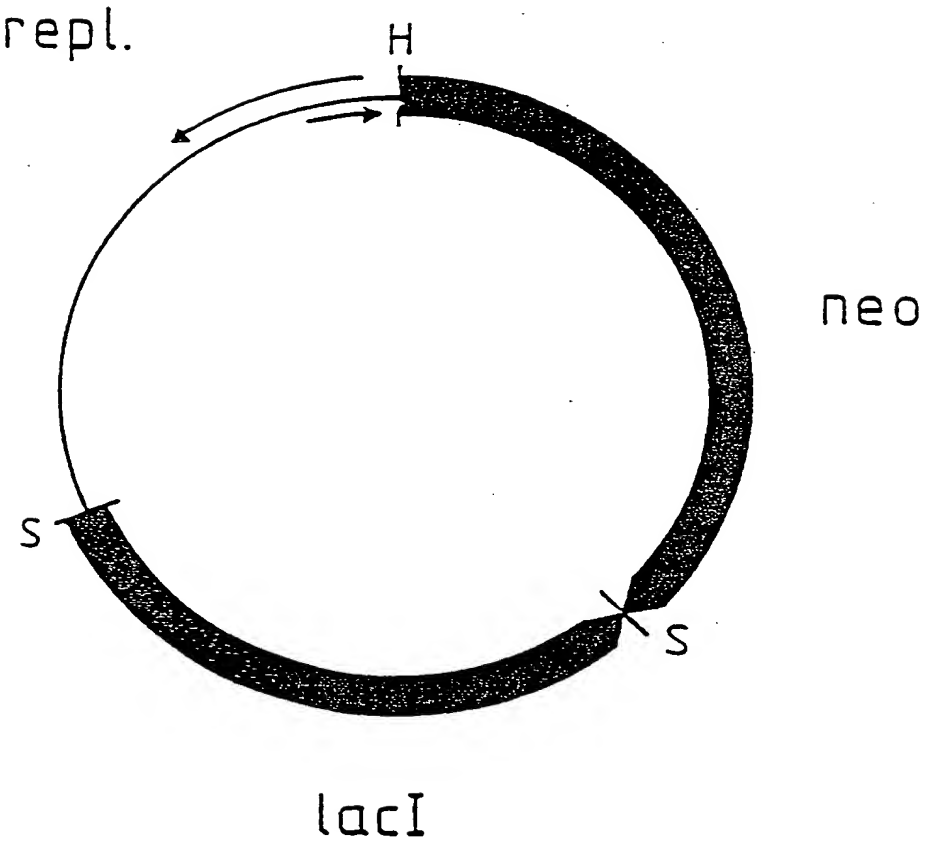

FIG. 2B/1

HindIII

```
   1  AAGCTTCACG CTGCCGCAAG CACTCAGGGC GCAAGGGCTG CTAAAGGAAG
  51  CGGAACACGT AGAAAGCCAG TCCGCAGAAA CGGTGCTGAC CCCGGATGAA
 101  TGTCAGCTAC TGGGCTATCT GGACAAGGGA AAACGCAAGC GCAAAGAGAA
 151  AGCAGGTAGC TTGCAGTGGG CTTACATGGC GATAGCTAGA CTGGGCGGTT
 201  TTATGGACAG CAAGCGAACC GGAATTGCCA GCTGGGGCGC CCTCTGGTAA
 251  GGTTGGGAAG CCCTGCAAAG TAAACTGGAT GGCTTTCTTG CCGCCAAGGA
 301  TCTGATGGCG CAGGGGATCA AGATCTGATC AAGAGACAGG ATGACGGTCG
 351  TTTCGCATGC TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG
 401  GGTGGAGAGG CTATTCGGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT
 451  CTGATGCCGC CGTGTTCCGG CTGTCAGCGC AGGGGCGCCC GGTTCTTTTT
 501  GTCAAGACCG ACCTGTCCGG TGCCCTGAAT GAACTGCAGG ACGAGGCAGC
 551  GCGGCTATCG TGGCTGGCCA CGACGGGCGT TCCTTGCGCA GCTGTGCTCG
 601  ACGTTGTCAC TGAAGCGGGA AGGGACTGGC TGCTATTGGG CGAAGTGCCG
 651  GGGCAGGATC TCCTGTCATC TCACCTTGCT CCTGCCGAGA AAGTATCCAT
 701  CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC
 751  CATTCGACCA CCAAGCGAAA CATCGCATCG AGCGAGCACG TACTCGGATG
 801  GAAGCCGGTC TTGTCGATCA GGATGATCTG GACGAAGAGC ATCAGGGGCT
 851  CGCGCCAGCC GAACTGTTCG CCAGGCTCAA GGCGCGCATG CCCGACGGCG
 901  AGGATCTCGT CGTGACCCAT GGCGATGCCT GCTTGCCGAA TATCATGGTG
 951  GAAAATGGCC GCTTTTCTGG ATTCATCGAC TGTGGCCGGC TGGGTGTGGC
1001  GGACCGCTAT CAGGACATAG CGTTGGCTAC CCGTGATATT GCTGAAGAGC
1051  TTGGCGGCGA ATGGGCTGAC CGCTTCCTCG TGCTTTACGG TATCGCCGCT
1101  CCCGATTCGC AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG
1151  AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC CCAACCTGCC
1201  ATCACGAGAT TTCGATTCCA CCGCCGCCTT CTATGAAAGG TTGGGCTTCG
1251  GAATCGTTTT CCGGGACGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC
1301  ATGCTGGAGT TCTTCGCCCA CCCCGGGCTC GATCCCCTCG CGAGTTGGTT
```

FIG 2B/2

```
1351  CAGCTGCTGC CTGAGGCTGG ACGACCTCGC GGAGTTCTAC CGGCAGTGCA
1401  AATCCGTCGG CATCCAGGAA ACCAGCAGCG GCTATCCGCG CATCCATGCC
                                                    SalI
1451  CCCGAACTGC AGGAGTGGGG AGGCACGATG GCCGCTTTGG TCGACAATTC
1501  GCGCTAACTT ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
1551  GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA
1601  GGCGGTTTGC GTATTGGGCG CCAGGGTGGT TTTTCTTTTC ACCAGTGAGA
1651  CGGGCAACAG CTGATTGCCC TTCACCGCCT GGCCCTGAGA GAGTTGCAGC
1701  AAGCGGTCCA CGCTGGTTTG CCCCAGCAGG CGAAAATCCT GTTTGATGGT
1751  GGTTAACGGC GGGATATAAC ATGAGCTGTC TTCGGTATCG TCGTATCCCA
1801  CTACCGAGAT ATCCGCACCA ACGCGCAGCC CGGACTCGGT AATGGCGCGC
1851  ATTGCGCCCA GCGCCATCTG ATCGTTGGCA ACCAGCATCG CAGTGGGAAC
1901  GATGCCCTCA TTCAGCATTT GCATGGTTTG TTGAAAACCG GACATGGCAC
1951  TCCAGTCGCC TTCCCGTTCC GCTATCGGCT GAATTTGATT GCGAGTGAGA
2001  TATTTATGCC AGCCAGCCAG ACGCAGACGC GCCGAGACAG AACTTAATGG
2051  GCCCGCTAAC AGCGCGATTT GCTGGTGACC CAATGCGACC AGATGCTCCA
2101  CGCCCAGTCG CGTACCGTCT TCATGGGAGA AAATAATACT GTTGATGGGT
2151  GTCTGGTCAG AGACATCAAG AAATAACGCC GGAACATTAG TGCAGGCAGC
2201  TTCCACAGCA ATGGCATCCT GGTCATCCAG CGGATAGTTA ATGATCAGCC
2251  CACTGACGCG TTGCGCGAGA AGATTGTGCA CCGCCGCTTT ACAGGCTTCG
2301  ACGCCGCTTC GTTCTACCAT CGACACCACC ACGCTGGCAC CCAGTTGATC
2351  GGCGCGAGAT TTAATCGCCG CGACAATTTG CGACGGCGCG TGCAGGGCCA
2401  GACTGGAGGT GGCAACGCCA ATCAGCAACG ACTGTTTGCC CGCCAGTTGT
2451  TGTGCCACGC GGTTGGGAAT GTAATTCAGC TCCGCCATCG CCGCTTCCAC
2501  TTTTTCCCGC GTTTTCGCAG AAACGTGGCT GGCCTGGTTC ACCACGCGGG
2551  AAACGGTCTG ATAAGAGACA CCGGCATACT CTGCGACATC GTATAACGTT
2601  ACTGGTTTCA CATTCACCAC CCTGAATTGA CTCTCTTCCG GGCGCTATCA
2651  TGCCATACCG CGAAAGGTTT TGCGCCATTC GATGGTGTCA ACGTAAATGC
```

FIG. 2B/3

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                                                    SalI
2701  ATGCCGCTTC GCCTTCGCGC GCGAATTGTC GACCCTGTCC CTCCTGTTCA
2751  GCTACTGACG GGGTGGTGCG TAACGGCAAA AGCACCGCCG GACATCAGCG
2801  CTAGCGGAGT GTATACTGGC TTACTATGTT GGCACTGATG AGGGTGTCAG
2851  TGAAGTGCTT CATGTGGCAG GAGAAAAAAG GCTGCACCGG TGCGTCAGCA
2901  GAATATGTGA TACAGGATAT ATTCCGCTTC CTCGCTCACT GACTCGCTAC
2951  GCTCGGTCGT TCGACTGCGG CGAGCGGAAA TGGCTTACGA ACGGGGCGGA
3001  GATTTCCTGG AAGATGCCAG GAAGATACTT AACAGGGAAG TGAGAGGGCC
3051  GCGGCAAAGC CGTTTTTCCA TAGGCTCCGC CCCCCTGACA AGCATCACGA
3101  AATCTGACGC TCAAATCAGT GGTGGCGAAA CCCGACAGGA CTATAAAGAT
3151  ACCAGGCGTT TCCCCTGGCG GCTCCCTCGT GCGCTCTCCT GTTCCTGCCT
3201  TTCGGTTTAC CGGTGTCATT CCGCTGTTAT GGCCGCGTTT GTCTCATTCC
3251  ACGCCTGACA CTCAGTTCCG GGTAGGCAGT TCGCTCCAAG CTGGACTGTA
3301  TGCACGAACC CCCCGTTCAG TCCGACCGCT GCGCCTTATC CGGTAACTAT
3351  CGTCTTGAGT CCAACCCGGA AAGACATGCA AAAGCACCAC TGGCAGCAGC
3401  CACTGGTAAT TGATTTAGAG GAGTTAGTCT TGAAGTCATG CGCCGGTTAA
3451  GGCTAAACTG AAAGGACAAG TTTTGGTGAC TGCGCTCCTC CAAGCCAGTT
3501  ACCTCGGTTC AAAGAGTTGG TAGCTCAGAG AACCTTCGAA AAACCGCCCT
3551  GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGCG CAGACCAAAA
3601  CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTC
3651  AGTGCAATTT ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT
3701  ATAAGTTGTT AATTCTCATG TTTGACAGCT TATCATCGAT
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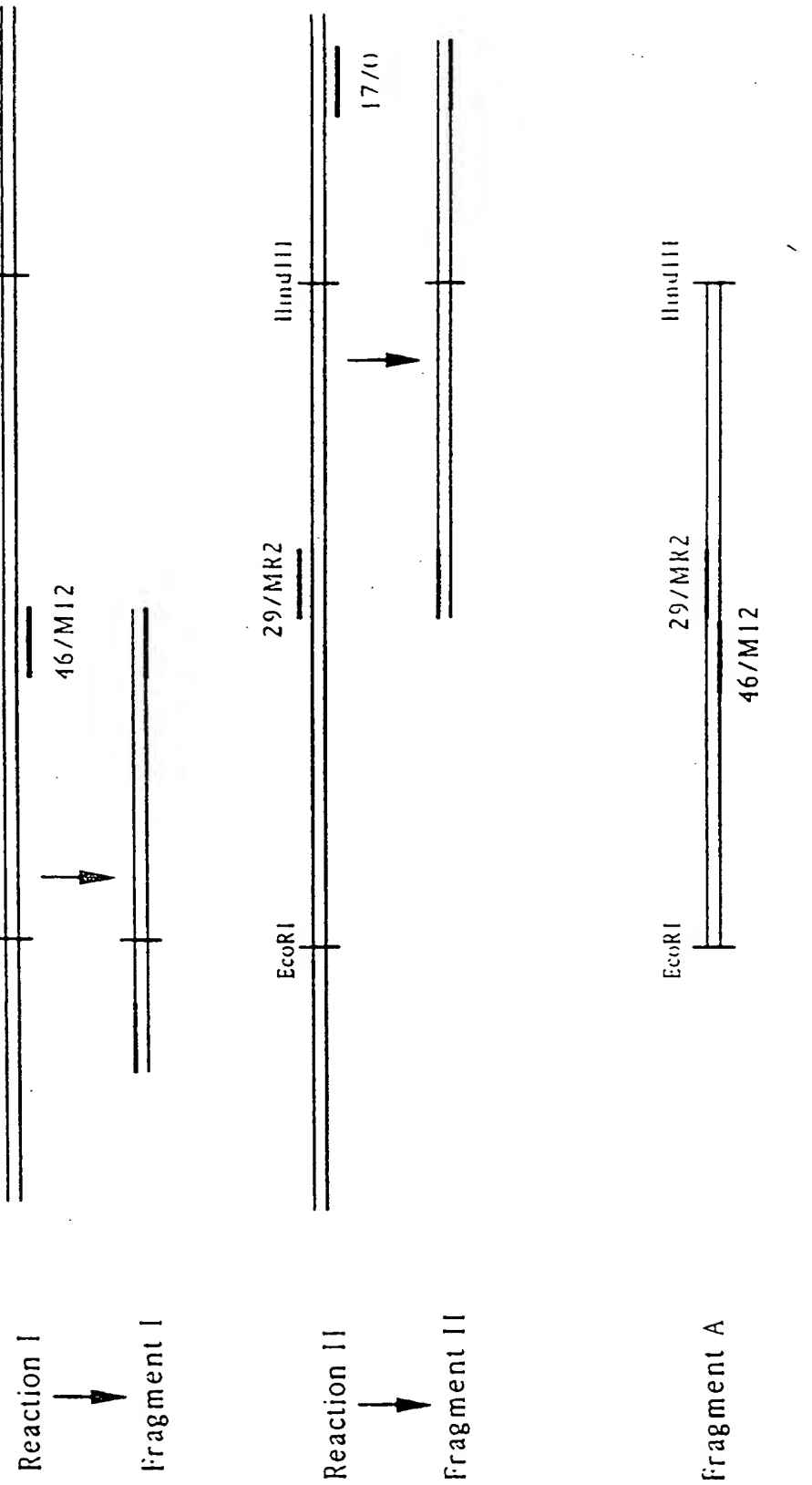
FIG. 3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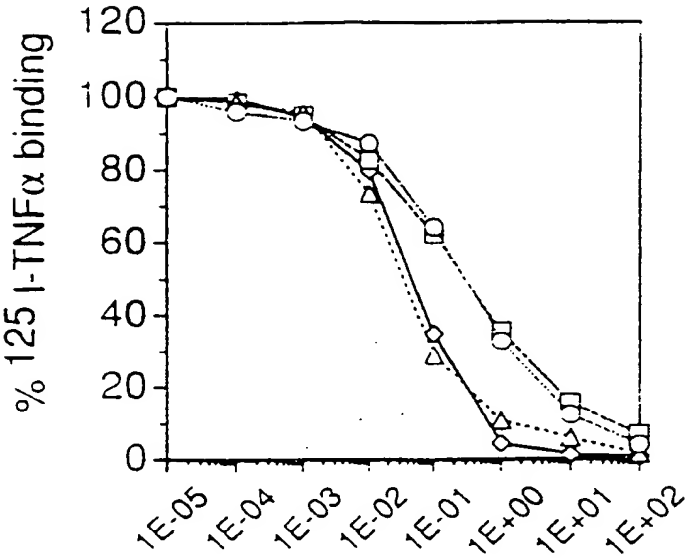
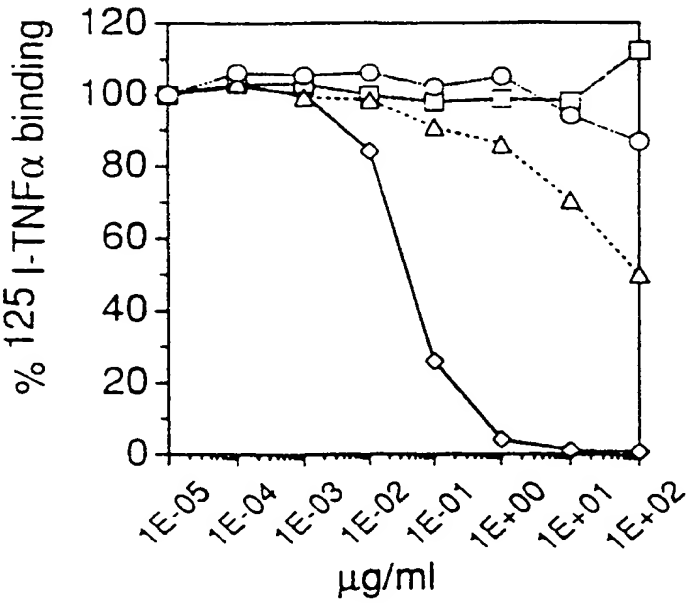
EcoRI
17/F
46/M12
Reaction I
Fragment I
HindIII
EcoRI
29/MR2
17/(
Reaction II
Fragment II
HindIII
EcoRI
29/MR2
46/M12
Fragment A

FIG. 4

TNFR-p75



TNFR-p55



European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 94 10 4154

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X	PROTEIN ENGINEERING vol. 4, no. 4 , April 1991 , ENGLAND GB pages 385 - 389 C. R. GOH ET AL 'Structural and functional domains in human tumour necrosis factors' * the whole document especially table I * ---	1-7	C12N15/28 C12P21/02 C07K13/00 A61K37/02 G01N33/68 C12N1/21
D,X	EP-A-0 168 214 (GENENTECH, INC.) * page 63, line 25 * ---	1-4, 9-13,15, 16	
D,X	EP-A-0 251 037 (DAINIPPON PHARMACEUTICAL CO.,LTD.) *the whole document especially the claims, example 9 ,page42 lines 29-31,page 44 lines 1-6 , page 25 table 5 * --- -/--	1-4	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)
C07K C12N

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	12 July 1994	Le Cornec, N

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 94 10 4154

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	PROTEIN ENGINEERING vol. 3, no. 8 , August 1990 , ENGLAND GB pages 713 - 719 JUN-ICHI YAMAGISHI ET AL 'Mutational analysis of structure-activity relationships in human Tumour Necrosis Factor-alpha' * the whole document * ---	1-4	
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 268, no. 35 , 15 December 1993 , BALTIMORE, MD US pages 26350 - 26357 H. LOETSCHER ET AL 'Human Tumour Necrosis Factor-alpha(TNF-alpha) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors' * the whole document * -----	1-7	TECHNICAL FIELDS SEARCHED (Int.Cl.5)

EPO FORM 1503 03.82 (P04C10)

EP 94 10 4154

-C-

Remark: Although claim 16 is directed to a method of treatment of (diagnostic method practised on) the human/animal body (Art. 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/ composition